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Phylogeny of Gyrinidae and Hydradephaga (Insecta: Coleoptera) based on CO Ⅰ gene: a case study using codon-partitioning schemes in phylogenetic tree reconstruction

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Abstract: Codon partition schemes were compared in phylogenetic analyses at subfamily level for Gyrinidae and at family level for Hydradephaga based on partial mitochondrial CO Ⅰ gene sequences. The results consistently demonstrated superiority for second codon partition data in analyses using ME, MP, or ML algorithms implemented in PAUP and Bayesian algorithm implemented in MrBayes. Optimal trees were inferred using PAUP based on second codon partition data and using MrBayes based on combined codon data, which showed good consistency and were consistent with the trees inferred from corresponding translated amino acid data. All analysis placed the Enhydrinae species *Porrorhynchus landaisi landaisi* in the unexpected most basal position of the Hydradephaga phylogenetic tree, which was supported by SH tests, justifying this species a monotypic family within Hydradephaga. In the ML tree based on the second codon partition data of the Hydradephaga dataset, monophyly of the whole Hydradephaga was well supported and Haliplidae and Dytiscoidea (= Dytiscidae, Hygrobiidae, Noteridae, and Amphizoidae) appeared as sister monophyletic groups. Topology of the trees was in accordance with the polyphyly hypothesis postulating three independent transitions to the aquatic environment. Mitochondrial DNA clock analysis suggested the radiation within a relatively short period of time (0.01 – 1.81 mya) of five pairs of closely related species that are currently distributed indifferent geographic regions.

Key words: Gyrinidae; mitochondrial CO Ⅰ gene; phylogenetic relationship; Hydradephaga

1 INTRODUCTION

The aquatic beetle suborder Hydradephaga comprises about 5 500 species in more than 200 genera and is subdivided into seven families. Gyrinidae is the second largest family in the suborder with about 1 000 species in 13 genera. The other families of the suborder are the diving beetle (Dytiscidae) with about 4 000 species and nine subfamilies, the burrowing water beetle (Noteridae) with 270 species, the crawling water beetle Haliplidae with 220 species, the squeak beetle (Hygrobiidae) with 6 species, the troutstream beetle (Amphizoidae) with 6 species, and Aspidytidae with one species (Ribera *et al*., 2002a).

Early in 1899, Gyrinidae was considered as one of the most distinct families of Coleoptera and was excluded from Hydradephaga (Sharp, 1899). It was considered to have originated from a pre-carabid ancestor with affinities to Palpicornia rather than being closely related to any of the existing groups of Coleoptera (Ochs, 1929). Lawrence and Newton (1982) considered that Gyrinidae ancestry comes from the distinct structure of *Spanglerogyrus*, which differs from all other gyrinids, and this links Gyrinidae with a more primitive type of Hydradephaga and makes it less likely that they have evolved from a dytiscoid. It was also suggested that Hydradephaga might be a polyphyletic group with various carabid or pre-carabid ancestors and Gyrinidae might have evolved independently from an early caraboid stock (Balfour-Browne, 1950). Several authors have recently proposed the polyphyly hypothesis postulating three independent transitions to the aquatic environment (Bell, 1966; Beutel and Roughley, 1988; Beutel, 1995; Beutel and Haas, 1996,). According to this hypothesis, Gyrinidae is the most primitive family of Hydradephaga, and beetles in this family represented an independent invasion of the water from other water beetles (Beutel and Roughley, 1988), followed by two further invasions

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by ancestors of Haliplidae (Hammond, 1979; Kavanaugh, 1986) and, independently, Dytiscoidea (= Dytiscidae, Hygrobiidae, Noteridae, and Amphizoidae). However, recent molecular studies showed that Hydradephaga was well supported as a monophyletic group (Shull *et al*., 2001; Ribera *et al*., 2002b; Balke *et al*., 2005), denying the hypothesis of multiple origin of Hydradephaga.

Traditionally, the family Gyrinidae is divided into three subfamilies: Gyrininae, Enhydrinae, and Orectochilinae, and has the greatest species diversity in the tropics and subtropics and tends to form local or geographical forms (subspecies). According to a catalogue of Chinese Gyrinidae, compiled by Jäch and Ji (1995) following Holmen's nomenclature (Holmen, 1987) for the subfamily and genera names, the family Gyrinidae includes three subfamilies: the subfamily Gyrininae with three genera *Gyrinus*, *Aulongyrus*, and *Metagyrinus*, the subfamily Enhydrinae with two genera *Dineutus* and *Porrorhynchus*, and the subfamily Orectochilinae with a monotypic genus *Orectochinus* consisting of two subgenera *Orectochinus* and *Partrus*.

Roughley (2001) mentioned that Gyrinidae consisted of two subfamilies, Spanglerogyrinae and Gyrininae, the latter including three tribes, Gyrinini, Enhydrini, and Orectochilini. Within Gyrininae, Orectochilini is the sister-group to Enhydrini plus Gyrinini. However, this taxonomy is not commonly accepted because the subfamily Spanglerogyrinae includes only one species *Spanglerogyrus*.

Beetles of the family Gyrinidae are rigid-bodied swimmers in circular patterns. Generally they form a very homogenous group occupying only a small niche surface in the aquatic habitat. The adult beetles are characterized by very much restricted morphological structures, which obviously do not indicate a recent evolution of the family, but reflect a response to the very restricted ecological and physiological conditions of the habitat (Vazirani, 1984), thus making it inappropriate to study the phylogeny of the family based on morphological characters alone. Although traditional classification and identification of insects that mainly depend on the morphological characters become more and more difficult, molecular markers were proved to be powerful tools in studies of phylogeny, evolution, and population dynamics (Loxdale and Hollander, 1989; Smith and Wayne, 1996). Phylogeny of hydradephagan water beetles inferred from 18s rRNA sequences suggested a basic group position of Gyrinidae within Hydradephaga (Ribera *et al*., 2002b). However, the intrafamilial relationships of the family Gyrinidae remained ambiguous and in disagreement with the results of existing morphological analysis (Belkaceme, 1991; Beutel and Roughley, 1994), *e.g*., *Macrogyrus* was placed within Orectochilini but not sister to *Andogyrus* (Enhydrini) and Dytiscoidea was recovered as monophyletic or paraphyletic group due to switch of the positions of Noteridae and Haliplidae.

In this study, our goal is to make further phylogenetic analysis of the Gyrinidae beetles using mitochondrial DNA (mtDNA) sequences. The phylogeny of Gyrinidae was based on sequence data for a part of the CO Ⅰ gene from 13 species representing the three subfamilies of Gyrinidae. We further analyzed phylogenetic relationship of the Hydradephaga at family level with the addition of sequence data of the same CO Ⅰ region retrieved from GenBank for 22 species representing the other six families of the suborder.

2 MATERIALS AND METHODS

2.1 Taxa, sequence data and saturation analysis

For Gyrinidae, partial CO Ⅰ sequence data for a total of 13 species, of which 9 were acquired by sequencing preserved specimens of Chinese Gyrinidae and 4 were retrieved from GenBank (Table 1), were included. The species represented all the three subfamilies of Gyrinidae, 6 of Orectochilinae, 3 of Gyrininae, and 4 of Enhydrinae. Two species of Dytiscidae, *Platambus obtusatus* and *P. princes*, were used as outgroups in the phylogeny analysis of Gyrinidae.

For analysis of the phylogeny of Hydradephaga at family level, CO Ⅰ partial sequences of the other six families were culled from GenBank, including 8 from Dytiscidae, 2 from Aspidytidae, 2 from Amphizoidae, 3 from Haliplidae, 4 from Noteridae, 3 from Hygrobiidae. Two haplotypes of a species of family Ptiliidae were included as outgroups. The outgroup taxa belong to Polyphaga and the infraorder was regarded as basal sister group of Adephaga (Lawrence and Newton, 1982).

The mitochondrial CO Ⅰ sequence is chosen for this study for several reasons: 1) Mitochondrial CO Ⅰ sequences have been widely used in taxonomic and population studies (Simon *et al*., 1994); 2) CO Ⅰ is relatively conservative, yet with enough variation for solving phylogenetic relationship at family to subfamily levels (Simon *et al*., 1994); 3) Earlier a mtDNA gene tree is considered to have an advantage over a gene tree derived from a single nuclear locus in tracking the population tree (Moore, 1995, 1997); and 4) Earlier attempts to acquire sequence data for several nuclear genes from old specimens were not successful.

The final data matrix consisted of 37 taxa. All sequences were cut to 744 bp long after alignment,

Table 1 List of taxa used in the phylogenetic trees in the study

No.	Taxa	Abbreviation	Source	Accession
	Family Ptiliidae			
1	*Acrotrichis xanthocera* haplotype F	*P. acroF*	USA	AY550882
2	*Acrotrichis xanthocera* haplotype A	*P. acroA*	USA	AY550854
	Family Haliplidae			
3	*Peltodytes rotundatus*	*H. peltodytes*	UK	AY071816
4	*Haliplus mucronatus*	*H. mucronatus*	UK	AY071804
5	*Haliplus lineatocollis*	*H. lineatocollis*	UK	AY071803
	Family Amphizoidae			
6	*Amphizoa insolens*	*A. insolens*	US	AY071796
7	*Amphizoa lecontei*	*A. lecontei*	US (California)	AY071797
	Family Aspidytidae			
8	*Aspidytes niobe* haplotype 1	*As. niobe*1	UK	AY071808
9	*Aspidytes niobe* haplotype 2	*As. niobe*2	UK	AY071809
	Family Dytiscidae			
	Subfamily Copelatinae			
10	*Aglymbus geotroi*	*A. geotroi*	Omen	AY138728
11	*Platambus princeps*	*P. princeps*	UK	AY138764
12	*Platambus obtusatus*	*P. obtusatus*	UK	AY138762
	Subfamily Coptotominae			
13	*Graphoderus cinereus*	*G. cinereus*	Spain	AY138735
	Subfamily Lancetinae			
14	*Lancetes lanceolatus*	*L. lanceolatus*	Australia	AY138760
15	*Lancetes varius*	*L. varius*	Chile	AY071810
	Subfamily Colymbetinae			
16	*Meladema coriacea*	*M. coriacea*	France	AF428209
17	*Colymbetes schildknechti*	*C. schildknechti*	Spain	AF428236
	Family Hygrobiidae			
18	*Hygrobia australiasiae*	*Hy. australiasiae*	Australia	AY071805
19	*Hygrobia hermanni*	*Hy. hermanni*	Spain	AY071806
20	*Hygrobia maculata*	*Hy. maculata*	Australia	AY071807
	Family Gyrinidae			
	Subfamily Gyrininae			
	Genus *Gyrinus*			
21	*Gyrinus minutus*[a*]	*G. minutus*	Nei Mongol, China	DQ266407[#]
22	*Gyrinus orientalis*[b*]	*G. orientalis*	Guangdong, China	DQ266406[#]
	Genus *Aulonogyrus*			
23	*Aulonogyrus striatus*[a*]	*A. striatus*	UK	AY071799
	Subfamily Orectochilinae			
	Subgenus *Orectochilus s. str.*			
24	*Orectochilus villosus villosus*[a*]	*O. villosus*	UK	AY071815
25	*Orectochilus fusiformis*[bc*]	*O. fusiformis*	Guangdong, China	DQ266405[#]
	Subgenus *Patrus*			
26	*Orectochilus dinghushanensis*[b*]	*O. ding*	Guangdong, China	DQ266403[#]
27	*Orectochilus Wui*[bc*]	*O. wui*	Guangdong, China	DQ266402[#]
28	*Orectochilus productus*[b*]	*O. productus*	Guangdong, China	DQ266404[#]
29	*Orectochilus chalceus*[bc*]	*O. chalceus*	Guangdong, China	DQ266401[#]
	Subfamily Enhydrinae			
	Genus *Dineutus*			
30	*Dineutus mellyi*[b*]	*D. mellyi*	Guangdong, China	DQ266408[#]
	Genus *Porrorhynchus*			
31	*Porrorhynchus landaisi landaisi*[b*]	*P. landaisi*	Hainan, China	DQ266409[#]
	Genus *Macrogyrus*			
32	*Macrogyrus* sp. IR-2001[a]	*M. IR*-2001	UK	AY071812
	Genus *Andogyrus*			
33	*Andogyrus ellipticus*[a]	*A. ellipticus*	UK	AY071798
	Family Noteridae			
34	*Suphisellus* sp. *IR*-2002	*N. suphisellus*	UK	AY071818
35	*Suphis* sp. *IR*-2002	*N. suphis*	UK	AY071817
36	*Noterus clavicornis*	*N. clavicornis*	UK	AY071814
37	*Notomicrus tenellus*	*N. tenellus*	UK	AY071813

Classification of Gyrinidae followed Jäch and Ji (1995): pp 155 – 172. [*] Species included in the present study. [#] Sequences acquired in this study (and the rest of the sequences were retrieved from GenBank). [a] Palearctic species, [b] Oriental species, [c] Distribution limited to China.

except for the Amphizoidae species sequence, which is 692 bp long. The matrix was analyzed as two datasets by selective inclusion of taxa: the Gyrinidae dataset consists of 15 sequences including 13 sequences from species of Gyrinidae and 2 from species of Dytiscidae as outgroup (Table 1), and the Hydradephaga dataset of 37 sequences including 35 sequences from species of Noteridae, Haliplidae, Dytiscidae, Aspidytidae, Amphizoidae, Hygrobiidae, Gyrinidae, and 2 from species of Ptiliidae as outgroup (Table 1). The aligned datasets may be downloaded from website: http://life.zsu.edu.cn/insect_tax/alignment_of_37_sequences.nex.

The nucleotide data matrix, nucleotide compositions, transition to transversion ratio, and the translated amino acid data were analyzed with MEGA ver. 2.1 package (Kumar *et al*., 2001). To evaluate saturation rates, the first, second and third codon positions were examined for possible saturation of transitions using plots of uncorrected *p* distance against Kimura 2-parameter (Gamma) distances (Kimura, 1980; Nei and Kumar, 2000). A gamma distribution (Γ) was assumed using a shape parameter $\alpha = 0.5$.

2.2 DNA extraction, amplification, and sequencing

Most specimens were from old collections in our laboratory of Entomology. Two fresh specimens were kept in 85% ethanol until DNA extraction. Only clean legs and thoracic muscles of individuals were used to avoid contamination with gut content or phoretic mites. Total DNA was extracted from single specimen using E.Z.N.A.(r) Insect DNA Kit (Omega bio-tek). PCR reaction was performed using primers C1-J- 2138 (Jerry) 5′-CAACATTTATTTTGATTTTTTGG-3′, and TL2-N-3014 (Pat) 5′- TCAATTGCACTAATCTGCC ATATTA-3′ (Simon *et al*., 1994), which contained 1.5 U Taq DNA polymerase, 10 mmol/L Tris-HCl, pH 9.0, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP. The following cycling conditions were generally used: started with denaturalization of 5 min at 94℃, followed by 33 cycles of 40 seconds at 95℃, 50 seconds at 45℃, 50 seconds at 72℃ and 8 min extension at 72℃. A CO Ⅰ gene sequence of approximate 878 bp was amplified. PCR products were purified in a 1% agarose gel using agarose gel extraction kit (Omega bio-tek) and were cloned to pT-Adv vector. The PCR sequencing using M13 forward and reverse primer was completed on ABI 377. Direct PCR sequencing was also performed. However, direct sequencing of the PCR products yielded uncertain characters especially at the terminal regions of the sequences, leading to a shorter usable length of the sequences. So we chose to clone the PCR products before sequencing.

Voucher specimens were deposited in the Institute of Entomology, Sun Yat-sen University. All nucleotide sequences newly acquired in this study have been submitted to GenBank (Table 1).

2.3 Phylogenetic analysis

All phylogenetic analysis were performed using PAUP* version 4.0b10 (Swofford, 2002) and MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Sequence alignments were carried out using Clustal X version 1.83. Gap sites were ignored in distance estimation by using the Pairwise-Deletion option. For protein-coding nucleotide sequences, a site-specific rate model was used. The codon site-partitioning schemes, *i. e.* first codon partition, second codon partition, first plus second codon partition, and combined codon data, were set up to allow each codon position to have its own rate, which resulted in various models of evolutionary distance for tree construction in the ME, MP, and ML analysis. In the MP analysis various site-specific rate models for partition datasets, including the first codon partition, the second codon partition, the first plus second codon partition, and combined codon data, weighted or unweighted, were set up (Table 5). The weighting schemes were used with estimated parameters for the first, second and third codon position based on the results of ts to tv ratio patterns and saturation rates inferred from plotting uncorrected *p* distance against k2p (gamma) model for the first, second and third codon position. As in MP analysis, minimum evolution (ME) trees were constructed from various partition data, using the tree-bisection-reconnection (TBR) branch-swapping algorithm. Distance measure "total character difference" was used and starting trees were obtained via neighbor joining. Negative branch lengths was allowed, but set to zero for tree-score calculation. In the ML analysis, the best ML model, a complex general time reversible (GTR) model with gamma distributed rate heterogeneity and an estimated proportion of invariable sites (a complex GRT + I + G model) were estimated with MrModeltest 2.0 (Nylander, 2004) using hierarchical likelihood ratio tests (hLRTs) and the Akaike Information Criterion (AIC).

Heuristic searches to find the ME, MP, and ML trees were performed with 10 random addition replicates and tree bisection-reconnection branch swapping. The starting tree was obtained via stepwise addition method. All trees were evaluated using the bootstrap test (Felsenstein, 1985) based on 1 000 replications for ME and MP methods, and 100 replications for ML method. The bootstrap analysis under a 50% majority rule resulted in bootstrap consensus trees. The consistency index (CI) and the retention index (RI) were also calculated. Branch support values (Decay indices) for MP method were estimated with program Autodecay V5.0 (Eriksson, 2001).

In MrBayes analysis, the evolutionary model was set

to the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. All other priors were the default values. Sampling frequency was every 100th generation. The analysis was stopped when the value of the standard deviation of split frequencies fell below 0.01 keeping adding generations. The tree values were summarized with 25% of samples, and tree files viewed by TreeView. The amino acid rate matrix prior used fixed or mixed rate models.

2.4 SH test

SH test (Shimodaira and Hasegawa, 1999), which is usually preferable when comparing many topologies, was performed using PAUP * 4.0b10 by comparing the hypotheses with the optimal ML tree. A null distribution of differences in log likelihoods was obtained by 10 000 replicates of nonparametric bootstrapping of reestimated log likelihoods with full likelihood optimization and a one-tailed test of significance. The species *Porrorhynchus landaisi landaisi* presented an unexpected position in all of the trees obtained. To assess the effect of recombination on the position of this species in more detail, in the process of assessing alternative hypothesis, we tested 11 topological constraints (Fig. 7 and Table 7) by using the SH test (Shimodaira and Hasegawa, 1999).

2.5 Molecular clock analysis

The molecular clock analysis was implemented using MEGA vs 2.1 (Kumar *et al*., 2001) based on Kimura-2 parameter (gamma) (Kimura, 1980) and excluding the informative third codon sites, with interior branch test. The relative rate constancy of a molecular clock was simultaneously tested using the same software. The time scale was calibrated using a rate of *ca*. 1.2% to 1.5% divergence per million years (Myr) per branch, which is commonly used for arthropod mtDNA (Dick *et al*., 2004) and has been calculated based on the estimates of the rates of pairwise sequence divergence of 1.2% – 1.3% per Myr for cave-dwelling Corsica-Sardinian beetles tectonically separated from the Iberian peninsula -29 million years ago (Mya) (Caccone and Sbordoni, 2001), and 1.5% per Myr for Tetraopes beetles whose origins coincide with the formation of the Sonoran desert (1 Mya) and aridification of the Southwest USA – 7 Mya (Farrell, 2001).

3 RESULTS

3.1 Sequence data and Saturation Analysis

For the Gyrinidae dataset, 228 characters (30.64%) were parsimony informative, among which 47 were at the first (6.32%), 16 at the second (2.15%), and 165 (22.17%) at the third codon positions. The ratio of transitions to transversions was 0.79 for the complete sequence. For the Hydradephaga dataset, 282 characters (37.9%) were parsimony informative, among which 65 (8.74%) were at the first, 22 (2.96%) at the second, and 194 (26.07%) at the third codon positions. The majority of parsimony informative characters were observed at the third codon position (165 or 194 bp). The ratio of transitions to transversions was 0.71 for all sites. The predominance of transitions mostly lay in the first codon (1.83 – 2.30) (Table 2).

Table 2 Data partition and indices observed for the Gyrinidae dataset (Gyrinidae + outgroup) and the Hydradephaga dataset (seven families of Hydradephaga + outgroup)

	Characters (n)	Ts to tv ratio	Variable sites/%	Pi site (n)/%	A	T	G	C	A + T
1st codon[1]	248	2.30	73/9.82	47/6.32	29.4	31.5	24.9	14.2	60.9
2nd codon[1]	248	0.49	27/3.63	16/2.15	20.5	41.0	14.9	23.6	61.6
3rd codon[1]	248	0.69	203/27.28	165/22.17	44.1	45.8	2.2	7.9	89.9
Total seq.[1]	744	0.79	303/40.73	228/30.64	31.3	39.4	14.0	15.3	70.7
aa[1]	248	–	58/23.39	39/15.73	–	–	–	–	–
1st codon[2]	248	1.83	87/11.69	65/8.74	29.1	31.5	25.2	14.2	60.6
2nd codon[2]	248	0.59	36/4.84	22/2.96	20.0	41.5	14.8	23.7	61.5
3rd codon[2]	248	0.61	225/30.24	194/26.07	43.3	46.7	2.4	7.6	90.0
Total seq.[2]	744	0.71	330/44.35	282/37.90	30.8	39.9	14.1	15.2	71.7
aa[2]	248	–	63/25.40	51/20.56	–	–	–	–	–

[1] The data processed from taxa of 15 sequnces data within ingroup Gyrinidae and the outgroup species, [2] The data processed from taxa of 37 sequences data within seven ingroup families: Amphizoidae, Aspidytidae, Dytiscidae, Haliplidae, Noteridae, Hygrobiidae, Gyrinidae, and outgroup family Ptiliidae. n refers to number; Pi refers to parsimony informative; % refers to percentage of ratio for data partition.

The nucleotide sequences were A/T rich, which was a common characteristic for insect mitochondrial DNA sequences. The average A + T base composition was 70.7% for the Gyrinidae dataset and 71.7% for the

Hydradephaga dataset. At the third codon position A + T content reached 90.0%.

When p distance for the overall taxa was plotted against Kimura 2-parameter (Gamma) distances (Kimura, 1980; Nei and Kumar, 2000) for all codon positions, minimal nucleotide saturation was observed at the first and second codon positions compared to high saturation at the third codon positions (Fig. 1).

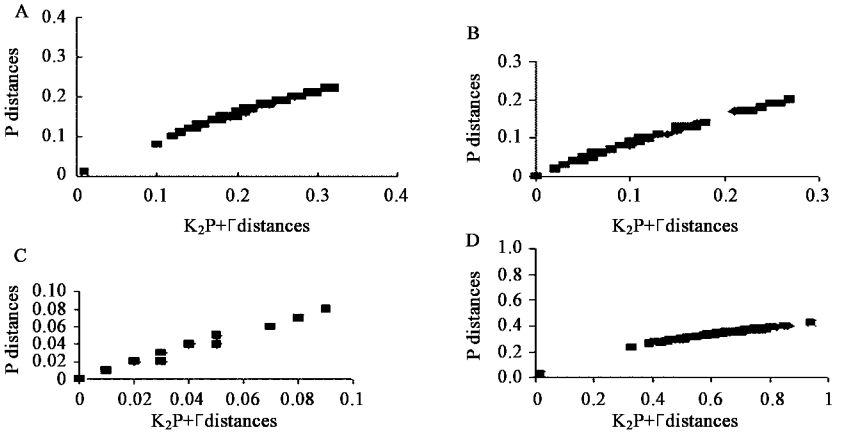

Fig. 1 Saturation analysis of the CO Ⅰ nucleotide substitution.
x axis: $K_2P+\Gamma$ distances; y axis: uncorrected P distances.
A: Scatter plot graphic for all codons; B – D: Scatter plot graphic for the first, the second, and the third positions in alignment, respectively.

3.2 Sequence divergence

Overall mean pairwise distances of partial CO Ⅰ sequences between or within subfamilies are calculated using Kimura 2-parameter (gamma) model (Kimura, 1980) in MEGA 2.1. Results were shown in Table 3 and Table 4 for the Gyrinidae and the Hydradephaga datasets, respectively.

The values from Table 3 suggested a close relation between subfamilies Enhydrinae and Gyrininae, and Orectochilinae was more closely related to Gyrininae than to Enhydrinae, which was in accordance with morphologic results (Beutel, 1990). Sequence divergence within subfamilies suggested a higher homogeneity of CO Ⅰ sequences inside subfamily Orectochilinae than within the other two subfamilies.

Table 3 Average distances among and within three subfamilies of Gyrinidae (excluding species *Porrorhynchus landaisi landaisi*)

	Orectochilinae	Gyrininae	Enhydrinae
Orectochilinae	0.1312		
Gyrininae	0.1757	0.1461	
Enhydrinae	0.1820	0.1542	0.1575

Average pairwise distances were gained by MEGA 2.1 using kimura 2-parameter (gamma) model. The data in lower left are distances among subfamilies of Gyrinidae. The diagonal boldfaced data are average distances within each subfamily. Taxa concerned are listed in Table 1.

Table 4 Average distance among and within nine groups including seven ingroup families, species *Porrorhynchus landaisi landaisi* and outgroup family Ptiliidae

	Aspidytidae	Amphizoidae	Dytiscidae	Gyrinidae	Haliplidae	Noteridae	Hygrobiidae	*P. landaisi*	Ptiliidae
Aspidytidae	0.0214								
Amphizoidae	0.1807	0.1205							
Dytiscidae	0.1964	0.2114	0.1897						
Gyrinidae	0.2325	0.2487	0.2580	0.1791					
Haliplidae	0.2057	0.2365	0.2171	0.2215	0.1413				
Noteridae	0.2583	0.2892	0.2768	0.2746	0.2428	0.2362			
Hygrobiidae	0.2277	0.2602	0.2558	0.2501	0.2324	0.2640	0.1407		
P. landaisi	0.2819	0.3246	0.2981	0.3179	0.2600	0.3267	0.2841	–	
Ptiliidae	0.3447	0.3745	0.3731	0.3623	0.3442	0.4060	0.3392	0.3512	0.0000

Average pairwise distances were gained by MEGA 2.1 using Kimura 2-parameter (gamma) model. The data in lower left are distances among groups. The diagonal boldfaced data are average distances within each group. Taxa used refer to Table 1. The species *Porrorhynchus landaisi landaisi* was excluded from Gyrinidae. *P. landaisi* is abbreviation to species *P. landaisi landaisi*.

Table 5 Results of parsimony analysis obtained from the codon site-partitioning scheme using the Gyrinidae dataset

Partition	Tree length	Number of trees saved	CI	RI
All codons	854	3	0.47	0.41
All codons (weight = 3:5:1)	977	2	0.49	0.43
All codons (weight = 1:3:1)	868	3	0.48	0.42
First + second codon	517	2	0.51	0.48
First + second codon (weight = 3:5)	643	1	0.53	0.49
First + second codon (weight = 1:3)	531	2	0.52	0.49
First codon	510	2	0.51	0.47
Second codon	465	1	0.51	0.49

Analysis of sequence divergence among families (Hydradephaga dataset) showed a close relationship among families Amphizoidae, Aspidytidae, and Dytiscidae, with corrected genetic distances of 18.07%, 19.64%, and 21.14 %, respectively (Table 4). Haliplidae was closely related to Aspidytidae and Dytiscidae with distance values of 20.5% and 21.7% respectively. The three remaining families, Noteridae, Hygrobiidae, and Gyrinidae, showed higher genetic distance values, ranging from 22.1% to 28.9%. The species *P. landaisi landaisi* was deviated from all families by genetic distance values ranging between 26% and 32%. The outgroup family Ptiliidae was distant from the ingroup families, with genetic distance values from 33.92% to 40.60%. The result was accordant with that of phylogenetic analysis. Sequence divergence within families suggested two major groups of Hydradephaga families: families with higher divergence degrees, including Noteridae (23.62%), Gyrinidae (17.91%), and Dytiscidae (18.97%), and families with lower divergence degrees, including Amphizoidae (12.05%), Haliplidae (14.13%), and Hygrobiidae (14.07%). It completely reflected the traditional taxonomy described by Crowson (1960).

A ME tree was constructed from the Gyrinidae dataset to estimate the divergence time of the species in Gyrinidae (Fig. 2), using MEGA vs 2.1 (Kumar *et al.*, 2001) based on Kimura-2 parameter (gamma) (Kimura, 1980) and excluding the third codon informative sites, with interior branch test.

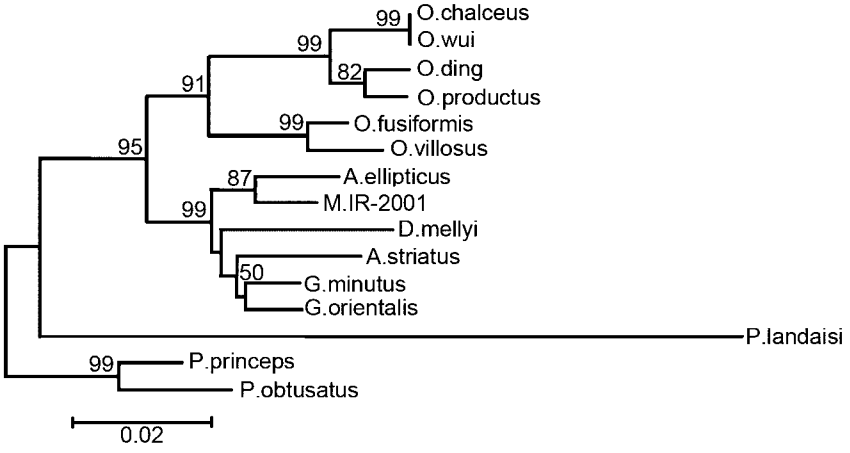

Fig. 2 Minimum Evolution tree based on mitochondrial CO Ⅰ genes of Gyrinidae, inferred by MEGA vs 2.1 (Kumar *et al.*, 2001) using Kimura-2 parameter (gamma) (Kimura, 1980) and with inrerior branch test of phylogeny, outgroup-rooted using the CO Ⅰ gene of *Platambus princes*, *Platambus obtusatus* from Dytiscidae
Numbers above branch refer to bootstrap value (>50%).

The rough divergence time was estimated for five pairs of closely related species: (i) divergence time of *Orectochilus chaleus* and *O. wui* with D = 0.0000 was not calculated; (ii) *O. dinghushanensis* and *O. productus* with D = 0.0122 was about 0.4083 – 0.5104 mya; (iii) *Orectochilus fusiformis* and *O. villosus* with D = 0.0165 was about 0.5510 – 0.6887 mya; (iv) *Andogyrus ellipticus* and *macrogyrus* sp. *IR* – 2001 with D = 0.0206 was about 0.6870 – 0.8588 mya; (v) *Gyrinus orientalis* and *G. minutus* with D = 0.0165 was about 0.5494 – 0.6868 mya. According to this result, most species within Gyrinidae diverged before Pleistocene Q1 (0.01 – 1.81 mya) based on the mitochondrial DNA clock.

3.3 Phylogenetic analysis of family Gyrinidae

MP, ME, and ML trees from the Gyrinidae dataset and ME and MP trees from the corresponding translated amino acid sequences data were constructed.

The best MP tree (length = 465 steps, CI = 0.51, RI = 0.49) (Fig. 3) was obtained for the second codon partition data. All the strict consensus trees from partition data excluding the third codon had similar topology, subfamily Gyrininae being recovered as monophyletic group, while subfamilies Orectochilinae and Enhydrinae being paraphyletic. The two clades of paraphyletic Orectochilinae corresponded to the two subgenera *patrus* and *Orectochilus s. str.* On the other hand, consensus trees from partition data containing the

third codon position, weighted or unweighted, reached ambiguous resolution and confused placement of species between subfamilies Enhydrinae and Gyrininae. To our surprise, in all the MP trees, the species *P. landaisi* *landaisi* from Enhydrinae appeared sister to other species of Gyrinidae with high bootstrap support (bootstrap value > 90%).

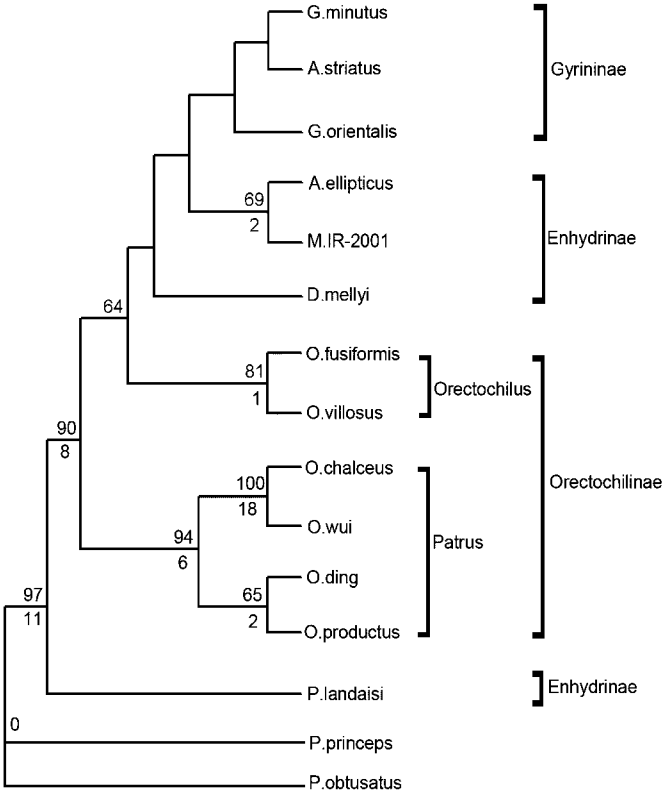

Fig. 3 Maximum parsimony tree obtained by PAUP based on the second codon partition of the Gyrinidae dataset. Numbers above branch refer to bootstrap values (> 50%). Decay indices above 1 are shown below branch. *Platambus btusatus* and *Platambus princeps* from Dytiscidae were used as outgroup. Interior vertical lines represent genus or subgenus, outside vertical lines represent subfamilies.

Best ME tree (ME score = 432) (Fig. 4) was also obtained for the second codon partition data. Orectochilinae and Gyrininae were successfully recovered as monophyletic groups with strong bootstrap support (96% and 83% respectively). The two subgenera of Orectochilinae, *patrus* and *Orectochilus s. st.*, were successfully resolved, though with poor bootstrap support (bootstrap value = 48%).

Enhydrinae remained paraphyletic and the species *P. landaisi landaisi* from Enhydrinae was also placed at basal position sister to other species of Gyrinidae with 98% bootstrap value. Consensus ME trees from other partition data also reached ambiguous resolution and confused placement of species among subfamilies Enhydrinae and Gyrininae.

Table 6 The Likelihood settings from the best-fit GTR + G + I model for various codon site-partitioning schemes selected by MrModeltest 2.2 using the Gyrinidae dataset

Partition	Base frequencies	Shape (α)	Pinvar	– lnL likelihood
Second codon	A = 0.2906, C = 0.1587, G = 0.1180, T = 0.4327	0.6666	0.4711	2638.1699
First codon	A = 0.3148, C = 0.1170, G = 0.1630, T = 0.4052	0.5963	0.4077	2794.2243
First plus second codon	A = 0.2282, C = 0.1411, G = 0.1619, T = 0.4089	0.6818	0.5172	3059.0735
Third codon	A = 0.4334, C = 0.0966, G = 0.0372, T = 0.4334	0.1573	–	3662.1460
All codons	A = 0.3555, C = 0.1066, G = 0.1223, T = 0.4156	0.2963	0.3081	4414.3944

In ML analysis, the models for partition data containing the third codon position showed much lower shape (α) values as compared to that for partition data excluding the third codon position among the various preferred GTR + G + I models (Table 6) for different partition data based on the results of MrModeltest and AIC evaluation. Best ML tree came from the second codon partition data (Fig. 5). Orectochilinae and

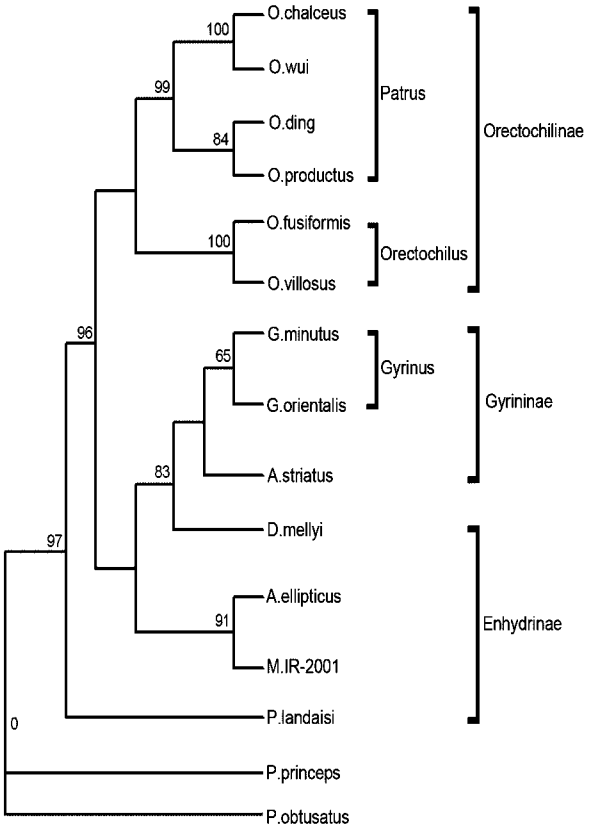

Fig. 4 Minimum evolution tree based on the second codon partition data of the Gyrinidae dataset by PAUP using total character difference.

Numbers above branch refer to bootstrap values (>50%). *Platambus btusatus* and *Platambus princeps* from Dytiscidae were used as outgroup. Interior vertical lines represent genus or subgenus, outside vertical lines represent subfamilies.

Table 7 Log-likelihood differences based on Shimodaira-Hasegawa test (SH test)

Alternative topologies	− ln L	Δln L	*P*-values
Assessment of the phylogenetic positions of *Porrorhynchus landaisi landaisi*			
Branch of the Optimal ML tree where the species is nested	5492.1666		
Enhydrinae of Gyrinidae (HYP 1)	5505.3279	13.1613	0.06
Gyrininae of Gyrinidae (HYP 2)	5507.4848	15.3181	0.03*
Orectochilinae of Gyrinidae (HYP 3)	5514.4607	22.2941	0.00*
Dytiscidae (HYP 4)	5503.7262	11.5595	0.07
Amphiziodae (HYP 5)	5515.6962	23.5296	0.00*
Aspidytidae (HYP 6)	5509.5155	17.3489	0.02*
Noteridae (HYP 7)	5507.7589	15.5923	0.04*
Hygrobiidae (HYP 8)	5508.3616	16.1949	0.04*
Haliplidae (HYP 9)	5508.0442	15.8775	0.02*
Between Haliplidae and Dytiscoidea (HYP 10)	5500.7588	8.5921	0.11
Dytiscoidea (HYP 11)	5504.6570	12.4904	0.06

Using the Shimodaira-Hasegawa (SH) test, we simultaneously compared the optimal ML tree to the constrained topologies that resulted from various hypotheses, based on the second codon partition data of the 37 sequences dataset. *, Topologies that are significantly rejected (*P*-values < 0.05). All hypotheses are described in Fig. 7. The following abbreviations are used: − lnL, negative natural logarithmic likelihood value for the hypothesis; Δ lnL, difference from the optimal tree; HYP, hypothesis.

Gyrininae were recovered as monophyletic groups with strong bootstrap support (100% and 81%, respectively). The two subgenera of Orectochilinae, *patrus* and *Orectochilus s. st.*, were well resolved with high bootstrap support (bootstrap value > 80%). As in MP and ME analysis, consensus trees from other partition data showed confused placement of species between subfamilies Enhydrinae and Gyrininae, though monophyletic Orectochilinae was well supported by high bootstrap values in all ML trees.

Bayesian analysis using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) were performed to certify the results using PAUP. The GTR + G + I

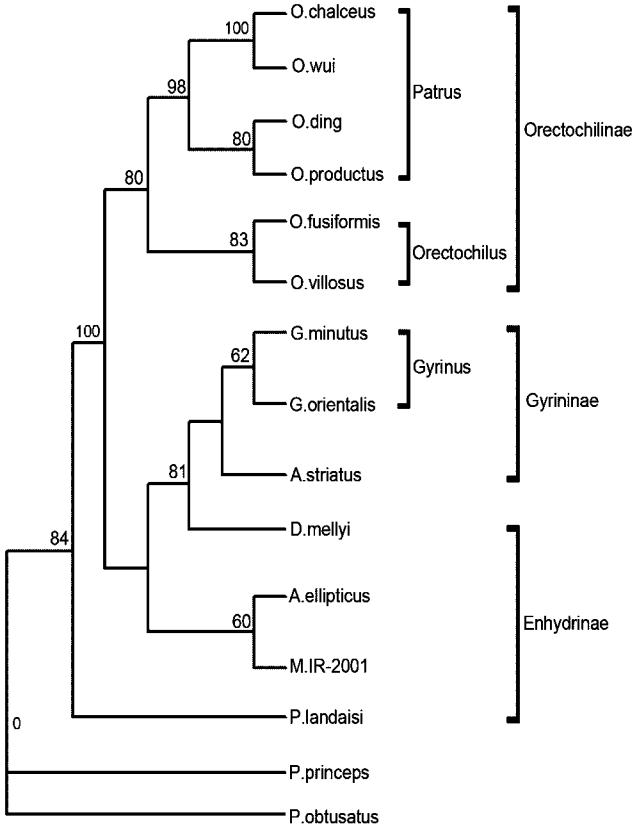

Fig. 5 Maximum likelihood tree based on the second codon partition data of the Gyrinidae dataset by PAUP using GTR + G + I model (− lnL = 2638.1699 ; A = 0.2906, C = 0.1587, G = 0.1180, T = 0.4327 ; gamma shape parameter = 0.6666 ; assumed proportion of invariable sites = 0.4711) .

Numbers above branch refer to bootstrap values (> 50%) . *Platambus btusatus* and *Platambus princeps* from Dytiscidae were used as outgroup. Interior vertical lines represent genus or subgenus, outside vertical lines represent subfamilies.

model was selected based on the results of MrModeltest and AIC evaluation. The resulted tree topology for combined codon data (tree not shown) was congruent with the topology of the best trees by PAUP. Bayesian analysis with F81 and HKY models resulted in similar tree topology (data not shown). However, Bayesian analysis with partitioned (site-specific) rate models led to conflicting results (data not shown).

MP, ME, and ML trees (trees not shown) inferred by PAUP or MrBayes from corresponding translated amino acid data were similar to the above optimal trees. The result was mostly congruent with morphological taxa (Jäch and Ji, 1995).

3.4 The phylogenetic relationships among the seven families of Hydradephaga

Phylogenetic trees were constructed based on the Hydradephaga dataset including 35 ingroup taxa and 2 outgroup taxa (Table 1) using the same codon-partitioning schemes as that used in Section 3.3. Results showed that the optimal tree topology and the best resolution also came from the second codon partition data. MP and ME analysis followed the above parameter sets (Section 3.3) and ML analysis used the GTR + G + I model (− lnL = 5 500.4398 ; A = 0.3650, C = 0.1137, G = 0.0452, T = 0.4761 ; gamma shape parameter = 0.4539 ; assumed proportion of invariable sites = 0.4942) .

MP and ME strict consensus trees were congruent : (Ptiliidae, (*P. landaisi landaisi*, (Hygrobiidae, (Noteridae, (Gyrinidae, (Haliplidae, (Amphizoidae, Aspidytidae, Dytiscidae (trees not shown). Topologies of MP and ME trees based on translated amino acid data matrix were similar to that based on the nucleotide acid data. Though monophyly of Hydradephaga and most families was recovered, the relationships among families were somewhat in conflict with the current taxonomy system (Beutel, 1995 ; Ribera *et al*., 2002b), Hygrobiidae being outside the clade of Dytiscidae plus Amphizoidae.

The tree topology obtained from ML analysis based on the second codon partition data (Fig. 6) agreed most with the current classification (Balke *et al*., 2005). Hydradephaga was recovered as monophyletic and *P. landaisi landaisi* plus the remaining species of Gyrinidae were located at the base of the tree. Excluding the species *P. landaisi landaisi*, Gyrinidae

formed a well-supported monophyletic group (bootstrap value = 65%), the three subfamilies presented a hierarchy arrangement of ((Gyrininae + Enhydrinae) + Orectochilinae). Dytiscoidea (= Dytiscidae, Hygrobiidae, Noteridae, and Amphizoidae) was successfully recovered as a monophyletic group. Hygrobiidae and Noteridae formed sister groups located at the base of Dytiscoidea. Aspidytidae was sister to Amphizoidae plus Dytiscidae, and Amphizoidae was sister to Dytiscidae.

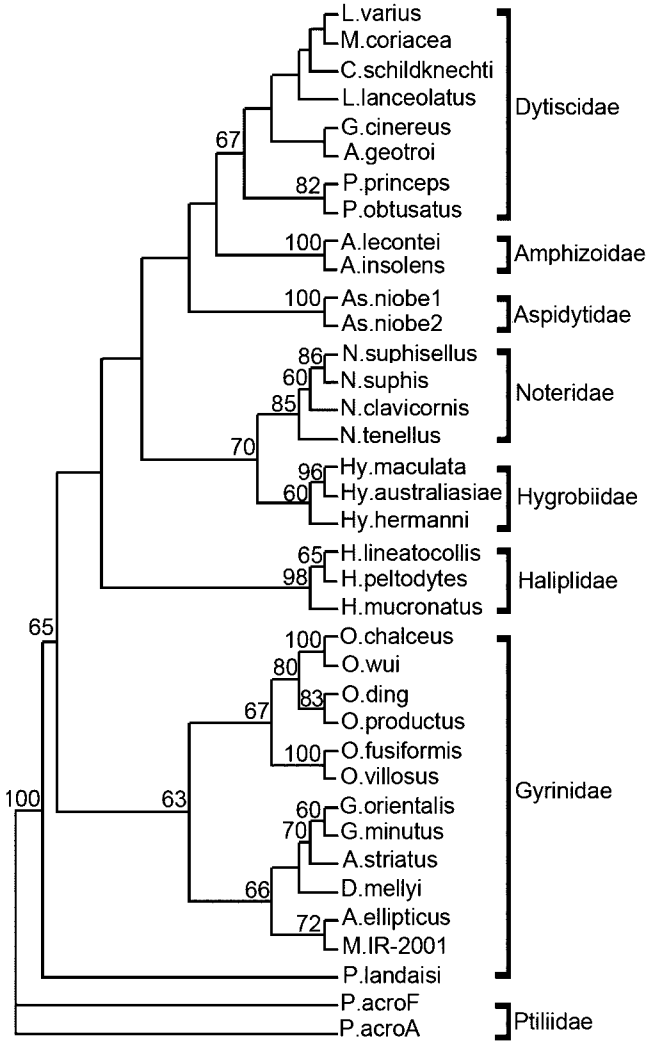

Fig. 6 The optimal ML tree by PAUP based on the second codon partition of the Hydradephaga dataset. Numbers above branch refer to bootstrap values (>50%). *P. acroA and P. acroF* from Ptiliidae were used as outgroup. Vertical lines represent families within Hydradephaga.

3.5 Alternative hypotheses tests (SH test) based on the phylogenetic position of the species Porrorhynchus landaisi landaisi and seven families within Hydradephaga

Results of the SH test showed that the minimum log-likelihood for the trees constrained by HYP (Hypothesis) 2, 3, 5, 6, 7, 8, and 9, in which the species *P. landaisi landaisi* was nested within Gyrininae of Gyrinidae, Orectochilinae of Gyrinidae, Amphizoidae, Aspidytidae, Noteridae, Hygrobiidae, and Haliplidae, respectively, were significantly lower than the optimal ML tree and significantly rejected in favor of the best tree (P-value < 0.05). The other four constraints, HYP 1, 4, 10, and 11, in which the species *P. landaisi landaisi* was nested within Enhydrinae of Gyrinidae, within Dytiscidae, between Haliplidae and Dytiscoidea, and within Dytiscoidea, could not be statistically rejected (*P*-value > 0.05). However, if 0.1 was taken as the significant *P*-value, all constraints except HYP 10 were rejected. And in HYP 10 the species *P. landaisi landaisi* was still located close to the basal position of the tree. The result indicated that the species *P. landaisi landaisi* should represent a family branching early in the tree.

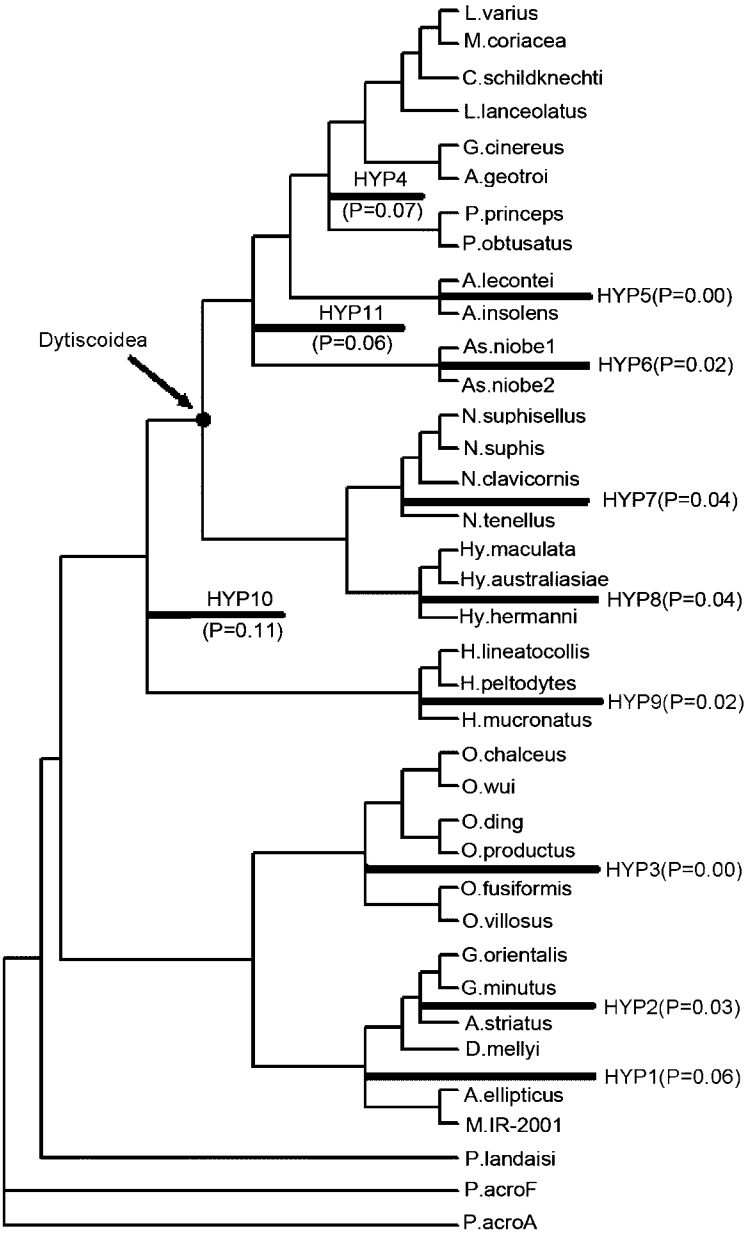

Fig. 7 Alternative hypotheses tests (SH – test) based on the position of species *P. landaisi*.
The optimal ML tree was obtained by PAUP based on the second codon partition of the Hydradephaga dataset.
The numbers inside brackets represent P-values; the broad-brushes represent constraining position of species *P. landaisi*;
HYP is abbreviation to Hypothesis. *P. landaisi* is abbreviation to species *Porrorhynchus landaisi landaisi*.

4 DISCUSSION

Previous studies have indicated that, in certain cases, the data are better explained by partitioning the dataset than by applying an average model and parameter values across codon positions (Nylander *et al*., 2004; Brandley *et al*., 2005; Mueller *et al*., 2004). In this study, codon site partitioning schemes were set up to analyze the phylogenetic relationships between species in Gyrinidae at subfamily level and species in Hydradephaga at family level. Results showed that the software PAUP was more affected than MrBayes by codon site partitioning schemes. This may be related to the algorithm of Bayesian methods that do not allow for sophisticated searches of parameter spaces with partitioned models (Yang, 1996). Although the second codon site partition contained the least parsimony informative characters, only 2.15% in the Gyrinidae dataset and 2.96% in the Hydradephaga dataset, ME, MP, and ML methods based on the second codon site partition data yielded better trees than that based on other partitioning strategies.

In the phylogenetic analysis of family Gyrinidae,

most optimal trees lead to the same conclusion that, among the three subfamilies, Orectochilinae and Gyrininae were resolved as monophyletic groups with high support. *Orectochilus dinghushanensis*, a new species (unpublished data) from Orectochilinae was successfully grouped into subgenus *Patrus* in Orectochilinae. Enhydrinae was not well resolved, while *Andogyrus* and *Macrogyrus* were recovered as having sister relationship with bootstrap value > 60%. The species *P. landaisi landaisi* from Enhydrinae was placed at the base in all trees. Excluding this species, the tree topology was similar to that from Beutel (1990), *i.e.* phylogenetic analysis based on CO Ⅰ gene sequences gained the same subfamily-level relationship as that based on morphologic characters. And mitochondrial CO Ⅰ gene as molecular marker was useful for phylogenetic studies within Gyrinidae. It contains enough phylogenetic information at different taxonomic ranks. Saturation of the third codon and transition of the first codon may be overtaken by using more complex models, estimating the best model that fix the data set, and taking into account a site-specific rate model.

Main conflict between the results based on CO Ⅰ sequences and morphologic characters was the position of the species *P. landaisi landaisi*. Morphological taxonomy treated this species as a member of genus *Porrorhynchus* of subfamily Enhydrinae while the CO Ⅰ trees suggested that it is monophyletic basal branch of the family tree. The result based on *CO* Ⅰ sequences was supported by SH test. When applying a 0.1 significant *P*-value, SH test rejected all constrains that placed this species within any family or subfamily. Only in one constrain was the species placed at a different position at the base of the tree. Even in this case, this species was not nested within any family, suggesting that the species *P. landaisi landaisi* represent a clade branching early in the tree.

The hypothesis is partly supported by the significant morphological differences of this species to other members of Enhydrinae: (a) Scutus disappeared, (b) Antennae with 2 + 8, others with 2 + 6, (c) Upper labium appears triangle with vertical length more than horizontal width, (d) 1/3 behind elytra like serration with 2-3 spines, (e) Elytra without sutural striae. Further morphological, behavioral, and molecular studies are necessary to test this hypothesis.

An alternative hypothesis for the unexpected position of *P. landaisi landaisi* is that the sequence amplified for this species was a nuclear mitochondrial pseudo gene (numt), which caused the unexpected result, bringing the species to the basal position of the tree. However, we precluded this possibility carefully at the sequence preparing stage, by using the universal primers; amplifying sequences from at least two insect individuals; carrying out DNA extraction, PCR, and sequencing procedures independently; PAGE, sequencing, and blastn search confirmation of the PCR products; and translating the resulted sequences with the invertebrate mitochondrial genetic code to ensure lack of stop codons. In addition, Fukuda *et al*. (1985) reported that mitochondrial gene transposition to the nuclear genome renders numts nonfunctional pseudo genes, and results in much slower rate of substitution in nuclear pseudo genes than in mitochondrial genes due to the superior proofreading activity of the nuclear DNA. It was reported that the putative tuatara nuclear mitochondrial pseudo gene (numt) has an average of 17.5% G compared to 14.7% G in tuatara mtCYTB (cytochrome b). More tellingly, at third codon positions which are under reduced selective constraint because fewer mutations at those sites alter the amino acid, nCYTB has 10.8% G compared to 2.8% G in mtCYTB (Hay *et al*., 2004). In the present study, the sequence exhibited an average of approximately 14.0% G in CO Ⅰ gene and approximately 2.2% G at third codon positions (see Table 2), which is consistent with the character of the mitochondrial CYTB gene.

The position of the family Gyrinidae within suborder Hydradephaga has long been resolved. The consensus viewpoint is that Gyrinidae is placed in the basal position of Hydradephaga. However family-level relationships within Hydradephaga are highly contentious, though Hydradephaga could be taken as a well-supported monophyletic group. Kavanaugh (1986) did not resolve the Dytiscoidea clade. Beutel (1995), on the basis of adult and larval characters, resolved Dytiscoidea as a monophyletic clade (Noteridae, Amphizoidae, Hygrobiidae, and Dytiscidae). Ribera *et al*. (2002b), the first work based on 18S rRNA, obtained similar result, except that Hygrobiidae was grouped within, rather than sister to Dytiscidae. Combining morphology and molecular data including 18s, 16s, 12s, H3, and CO Ⅰ, Balke *et al*. (2005) also resolved the Dytiscoidea clade in which Hygrobiidae was outside the subclade (Amphizoidae + Dytiscidae), similar to our result from this study (Fig. 6).

Another controversy, whether the ancestral Hydradephagan is aquatic or terrestrial, is also resolved. It is commonly accepted that the Hydradephaga are ancestrally terrestrial (Lawrence and Newton, 1982), Gyrinidae being the first invasion to the aquatic environment, followed by two further invasions by ancestors of Haliplidae (Hammond, 1979; Kavanaugh, 1986) and, independently, Dytiscoidea

(= Dytiscidae, Hygrobiidae, Noteridae, and Amphizoidae). This hypothesis is supported by the results of the present study. The optimal ML tree (Fig. 6) showed three obvious main clades, Gyrinidae, Haliplidae, and Dytiscoidea. Gyrinidae was monophyletic at the base of Hydradephaga, and monophyletic Haliplidae was sister to Dytiscoidea (= Dytiscidae, Hygrobiidae, Noteridae, and Amphizoidae) in which each family was also recovered as monophyletic. This result reflects the phylogenetic relationships of the three origins.

However, several recent molecular studies challenged the multiple-origin hypothesis for the Hydradephaga and suggested that Hydradephaga is a well-supported monophyletic group (Shull *et al*., 2001; Ribera *et al*., 2002b; Balke *et al*., 2005). This hypothesis is supported by the results of our study. These results indicated Hydradephaga are a monophyletic group that were ancestrally terrestrial, which explains the monophyly result of the molecular studies.

Analysis of the mitochondrial DNA clock assumed that most species of Gyrinidae were divergent before Pleistocene Q1 (0.01 – 1.81 mya), which was in accordance with the fact that many beetles in amber were found from Pliocene N2 (1.81 – 5.32 mya) to Pleistocene Q1 (0.01 – 1.81 mya) (http://www.fossilmuseum.net/). These modern species of Gyrinidae might have radiated within a short period and diverged according to their geographic distributions. However, Hydradephagans might have diverged from their sister group in the late Permian, with the most recent common ancestor of living Hydradephagans probably existing in the early Triassic, around 240 million years ago (Erwin, 1979).

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豉甲科及水生肉食亚目的分子系统发育学分析
（昆虫纲 鞘翅目）

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摘要：利用 PAUP 和 MrBayes 软件，对线粒体 CO Ⅰ 基因序列 3 个密码子位置的数据模块分别进行了豉甲科（Gyrinidae）和水生肉食亚目（Hydradephaga）在亚科或科水平上的系统发育学分析，结果表明第二密码子数据模块获得了理想的分析结果。由 PAUP 生成的豉甲科最优树来自第二密码子数据模块的分析，而由 MrBayes 生成的最优树来自全部密码子数据模块的分析。此外，用对应的氨基酸序列生成的 ME 和 MP 树与第二密码子数据模块分析的结果也一致。亚科 Orectochilinae 和 Gyrininae 以高的支持率形成了单系。然而，来自亚科 Enhydrinae 的种 *Porrorhynchus landaisi landaisi* 呈现了异常的位置。SH-test 检验也支持该异常位置，表明这个种可能代表了一个科。在来自第二密码子数据模块的水生肉食亚目最优 ML 树中，整个 Hydradephaga 树呈现单系，豉甲科位于树的基部，表明了该科在水生肉食亚目中是一个早期的分支。在树中还产生了一个单系的 Dytiscoidea 总科，由 Dytiscidae、Hygrobiidae、Noteridae 和 Amphizoidae 4 个科组成，单系的 Haliplidae 与之成为姐妹群。此外线粒体分子钟的结果表明豉甲科的 5 对相近种间的分化是一个短时期内发生的（0.01 ~ 1.81 百万年前），这点可能与它们的特殊地理分布有关。

关键词：豉甲科 线粒体 CO Ⅰ 基因 系统发育学 水生肉食亚目

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